invitrogen™

pCR®8/GW/TOPO® TA Cloning® Kit

Five-minute, TOPO® Cloning of *Taq* polymerase-amplified PCR products into an entry vector for the Gateway® System

Catalog nos. K2500-20, K2520-20, and K2520-02

Version E
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User Manual

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TOPO® Cloning Procedure for Experienced Users

Introduction

This quick reference sheet is provided for experienced users of the TOPO® Cloning procedure. If you are performing the TOPO® Cloning procedure for the first time, we recommend that you follow the detailed protocols provided in the manual.

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Produce PCR product</td><td>Produce PCR products using Taq polymerase and your own protocol. End the PCR reaction with a final 7 to 30 minute extension step.</td></tr>
<tr><td>Perform the TOPO® Cloning Reaction</td><td>1. Set up one of the following TOPO® Cloning reactions using the reagents in the order shown. For electroporation, dilute Salt Solution 4-fold to prepare Dilute Salt Solution.

<table>
<tr><th>Reagent</th><th>Chemical Transfection</th><th>Electroporation</th></tr>
<tr><td>Fresh PCR product</td><td>0.5 to 4 µl</td><td>0.5 to 4 µl</td></tr>
<tr><td>Salt Solution</td><td>1 µl</td><td>--</td></tr>
<tr><td>Dilute Salt Solution</td><td>--</td><td>1 µl</td></tr>
<tr><td>Water</td><td>to a final volume of 5 µl</td><td>to a final volume of 5 µl</td></tr>
<tr><td>TOPO® Vector</td><td>1 µl</td><td>1 µl</td></tr>
<tr><td>Total volume</td><td>6 µl</td><td>6 µl</td></tr>
</table>
2. Mix gently and incubate for 5 minutes at room temperature.
3. Place on ice and proceed to transform One Shot® chemically competent E. coli, below.</td></tr>
<tr><td>Transform One Shot® Chemically Competent E. coli</td><td>1. For each transformation, thaw one vial of One Shot® E. coli cells on ice.
2. Add 2 µl of the TOPO® Cloning reaction into a vial of One Shot® chemically competent E. coli and mix gently.
3. Incubate on ice for 5 to 30 minutes.
4. Heat-shock the cells for 30 seconds at 42°C without shaking. Immediately transfer the tube to ice.
5. Add 250 µl of room temperature S.O.C. Medium.
6. Incubate at 37°C for 1 hour with shaking.
7. Spread 10-50 µl of bacterial culture on a prewarmed LB agar plate containing 100 µg/ml spectinomycin, and incubate overnight at 37°C.</td></tr>
</table>

Control Reaction

We recommend using the Control PCR Template and the Control PCR Primers included with the kit to perform the control reaction. See the protocol on pages 19-20 for instructions.

Kit Contents and Storage

Types of Kits

This manual is supplied with the following kits:

Kit	Catalog no.
pCR®8/GW/TOPO® TA Cloning Kit	
with One Shot® TOP10 Chemically Competent E. coli	K2500-20
with One Shot® Mach1™-T1^R Chemically Competent E. coli	K2520-20
with One Shot® Mach1™-T1^R Chemically Competent E. coli and PureLink™ Quick Plasmid Miniprep Kit	K2520-02

Shipping/Storage

Each pCR®8/GW/TOPO® TA Cloning® Kit is shipped on dry ice, and contains 2 or 3 boxes as described below. Upon receipt, store the boxes as detailed below.

Box	Component	Catalog no.			Storage
		K2500-20	K2520-20	K2520-02	
1	pCR®8/GW/TOPO® Reagents	√	√	√	-20°C
2	One Shot® Chemically Competent *E. coli*	√	√	√	-80°C
3	PureLink™ Quick Plasmid Miniprep Kit			√	Room temperature

continued on next page

Kit Contents and Storage, continued

pCR®8/GW/TOPO® Reagents

The following reagents are supplied with the pCR®8/GW/TOPO® vector (Box 1). **Note that the user must supply *Taq* polymerase. Store Box 1 at -20°C.**

Item	Concentration	Amount
pCR®8/GW/TOPO® vector, TOPO®-adapted	5-10 ng/µl linearized plasmid DNA in: 50% glycerol 50 mM Tris-HCl, pH 7.4 (at 25°C) 1 mM EDTA 1 mM DTT 0.1% Triton X-100 100 µg/ml BSA 30 µM phenol red	20 µl
10X PCR Buffer	100 mM Tris-HCl, pH 8.3 (at 42°C) 500 mM KCl 25 mM $MgCl_2$ 0.01% gelatin	100 µl
dNTP Mix	12.5 mM dATP 12.5 mM dCTP 12.5 mM dGTP 12.5 mM dTTP neutralized at pH 8.0 in water	10 µl
Salt Solution	1.2 M NaCl 0.06 M $MgCl_2$	50 µl
Water	---	1 ml
GW1 Primer	0.1 µg/µl in TE Buffer, pH 8.0	20 µl
GW2 Primer	0.1 µg/µl in TE Buffer, pH 8.0	20 µl
Control PCR Primers	0.1 µg/µl **each** in TE Buffer, pH 8.0	10 µl
Control PCR Template	0.05 µg/µl in TE Buffer, pH 8.0	10 µl

Primer Sequences

The table below provides the sequences of GW1 and GW2 primers. Note that the sequences of the GW1 and GW2 primers are identical except for the last 2 nucleotides at the 3′ end (indicated in bold).

Primer	Sequence	pmoles Supplied
GW1	5´-GTTGCAACAAATTGATGAGCAATGC-3´	260
GW2	5´-GTTGCAACAAATTGATGAGCAATTA-3´	260

continued on next page

Kit Contents and Storage, continued

One Shot® Reagents

The following reagents are included with the One Shot® TOP10 or Mach1™-T1^R Chemically Competent *E. coli* kit (Box 2). Transformation efficiency is ≥ 1 x 10^9 cfu/µg plasmid DNA. **Store Box 2 at -80°C.**

Reagent	Composition	Amount
S.O.C. Medium (may be stored at room temperature or +4°C)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 ml
TOP10 or Mach1™-T1^{R}cells	--	21 x 50 µl
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µl

Genotype of *E. coli* Strains

TOP10: F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

Mach1™-T1^R: F$^-$ Φ80*lac*ZΔM15 Δ*lac*X74 *hsd*R(r_k^-, m_k^+) Δ*rec*A1398 *end*A1 *ton*A (confers resistance to phage T1)

Information for Non-U.S. Customers Using Mach1™-T1^R Cells

The parental strain of Mach1™-T1^R *E. coli* is the non-K-12, wild-type W strain (ATCC #9637, S.A. Waksman). Although the parental strain is generally classified as Biosafety Level 1 (BL-1), we recommend that you consult the safety department of your institution to verify the Biosafety Level.

PureLink™ Quick Plasmid Miniprep Kit

For kit components of the PureLink™ Quick Plasmid Miniprep Kit (Box 3) supplied with cat. no K2520-02, refer to the manual supplied with the miniprep kit.

Accessory Products

Introduction

The products listed in this section may be used with the pCR®8/GW/TOPO® TA Cloning® Kit. For more information, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 27).

Additional Products

Some of the reagents supplied in the pCR®8/GW/TOPO® TA Cloning® Kit and other reagents suitable for use with the kits are available separately from Invitrogen. Ordering information for these reagents is provided below.

Note: Other reagent quantities may be available.

Item	Quantity	Catalog no.
Platinum® *Taq* DNA Polymerase	100 reactions 250 reactions 500 reactions	10966-018 10966-026 10966-034
Taq DNA Polymerase, Recombinant	100 units 250 units 500 units	10342-053 10342-012 10342-020
Platinum® *Taq* DNA Polymerase High Fidelity	100 units 500 units	11304-011 11304-029
One Shot® TOP10 Chemically Competent *E. coli*	10 reactions 20 reactions	C4040-10 C4040-03
One Shot® TOP10 Electrocompetent *E. coli*	10 reactions	C4040-50
One Shot® Mach1™-T1^R Chemically Competent *E. coli*	20 reactions	C8620-03
LB Broth	500 ml	10855-021
LB Agar	500 g	22700-025
PureLink™ Quick Plasmid Miniprep Kit	50 reactions	K2100-10
PureLink™ Quick Gel Extraction Kit	50 reactions	K2100-12
Gateway® LR Clonase™ II Enzyme Mix	20 reactions 100 reactions	11791-020 11791-100
Gateway® LR Clonase™ Plus Enzyme Mix	20 reactions	12538-013
MultiSite Gateway® Three-Fragment Vector Construction Kit	1 kit	12537-023

Spectinomycin

For selection of pCR®8/GW/TOPO® transformants in *E. coli*, you will need to obtain spectinomycin. Spectinomycin dihydrochloride is available from Sigma (Catalog no. S4014). For a recipe to prepare spectinomycin for use, see page 26.

Introduction

Overview

Introduction

The pCR®8/GW/TOPO® TA Cloning® Kit combines Invitrogen's TOPO® Cloning and Gateway® technologies to facilitate 5-minute, one-step cloning of *Taq* polymerase-amplified PCR products into a plasmid vector with ≥ 95% efficiency. As is the case with other pCR® vectors (*e.g.* pCR®2.1-TOPO®), clones may be easily sequenced and characterized. Once characterized, clones may also be transferred from the pCR®8/GW/TOPO® entry vector to a Gateway® or MultiSite Gateway® destination vector of choice for expression of the gene of interest in virtually any system.

For more information about how TOPO® Cloning works and the Gateway® and MultiSite Gateway® technologies, see the rest of this section.

Advantages of Using pCR®8/GW/TOPO®

Using the pCR®8/GW/TOPO® vector for cloning applications provides the following advantages:

- The vector is TOPO®-adapted to allow highly efficient, 5-minute cloning of *Taq* polymerase-amplified PCR products. No ligase, post-PCR procedures, or restriction enzymes are required.
- The vector contains primer binding sites that are located within 55 base pairs of the TOPO® Cloning site to facilitate sequencing of the PCR product while minimizing the amount of vector-encoded DNA that needs to be read.
- The vector is Gateway®-adapted to allow easy recombination-based transfer of the PCR product of interest into any Gateway® destination vector for downstream analysis.
- *Eco*R I sites flank the TOPO® Cloning to simplify excision of the cloned PCR product.
- The vector contains the spectinomycin resistance marker for efficient selection in *E. coli.* Use of this particular marker also allows recombination-based transfer of the PCR product into ampicillin- or kanamycin-resistant Gateway® destination vectors.

Features of the pCR®8/GW/TOPO® Vector

Features of the pCR®8/GW/TOPO® vector include:

- TOPO® Cloning site for rapid and efficient cloning of *Taq*-amplified PCR products (see the next page for more information)
- *att*L1 and *att*L2 sites for recombination-based transfer of the gene of interest into any Gateway® destination vector
- Specifically designed primer binding sites within the *att*L1 and *att*L2 sites for sequencing using the GW1 and GW2 primers
- *rrn*B transcription termination sequences to prevent basal expression of the PCR product of interest in *E. coli*
- Spectinomycin resistance gene for selection in *E. coli*
- pUC origin for high-copy replication of the plasmid in *E. coli*

continued on next page

Overview, continued

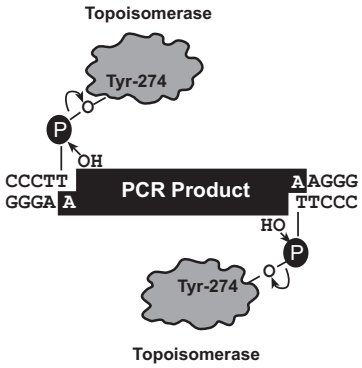
How Topoisomerase I Works

The pCR®8/GW/TOPO® vector is supplied linearized with:

- Single 3′-thymidine (T) overhangs for TA Cloning®
- Topoisomerase I covalently bound to the vector (referred to as "activated" vector)

Taq polymerase has a non-template-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3′ ends of PCR products. The linearized vector supplied in this kit has single, overhanging 3′ deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector.

Topoisomerase I from *Vaccinia* virus binds to duplex DNA at specific sites (CCCTT) and cleaves the phosphodiester backbone in one strand (Shuman, 1991). The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3′ phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5′ hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase (Shuman, 1994). TOPO® Cloning exploits this reaction to efficiently clone PCR products.



The Gateway® Technology

The Gateway® Technology is a universal cloning method that takes advantage of the site-specific recombination properties of bacteriophage lambda (Landy, 1989) to provide a rapid and highly efficient way to move your gene of interest into multiple vector systems. To express your gene of interest using the Gateway® Technology, simply:

1. TOPO® Clone your *Taq*-amplified PCR product into pCR®8/GW/TOPO® to generate an entry clone.
2. Generate an expression construct by performing an LR recombination reaction between the entry clone and a Gateway® destination vector of choice.
3. Introduce your expression construct into the appropriate host (*e.g.* bacterial, mammalian, yeast, insect) and express your recombinant protein.

For more information about the Gateway® Technology, refer to the Gateway® Technology with Clonase™ II manual which is available for downloading from www.invitrogen.com or by contacting Technical Service (see page 27).

continued on next page

Overview, continued

*att*L Sites and Sequencing

Inserts cloned into most Gateway® entry vectors (*e.g.* pENTR™/D-TOPO®) can be sequenced using M13 forward (-20) and M13 reverse primers. The M13 forward (-20) and M13 reverse primer binding sites are located upstream and downstream of the *att*L1 and *att*L2 sites, respectively, requiring that at least 130 base pairs of vector-encoded DNA be read before reaching the insert DNA. To facilitate more efficient sequencing and to minimize the amount of vector-encoded DNA that needs to be read, three nucleotides within the *att*L2 site of pCR®8/GW/TOPO® have been mutated. This results in the following:

- Allows robust and efficient sequencing of inserts cloned into pCR®8/GW/TOPO® using the GW1 and GW2 primers.
- The GW1 and GW2 primer binding sites are located within the *att*L1 and *att*L2 sites, thereby minimizing the amount of vector-encoded DNA that needs to be read to less than 55 base pairs (see the diagram on page 6 for the location of the primer binding sites).
- Does not affect the efficiency of LR recombination between pCR®8/GW/TOPO® and Gateway® destination vectors.

Note: The pCR®8/GW/TOPO® vector also contains the M13 forward (-20) and M13 reverse primer binding sites to allow sequencing using the M13 forward (-20) and M13 reverse primers, if desired. The T7 promoter/priming site is also present in the vector.

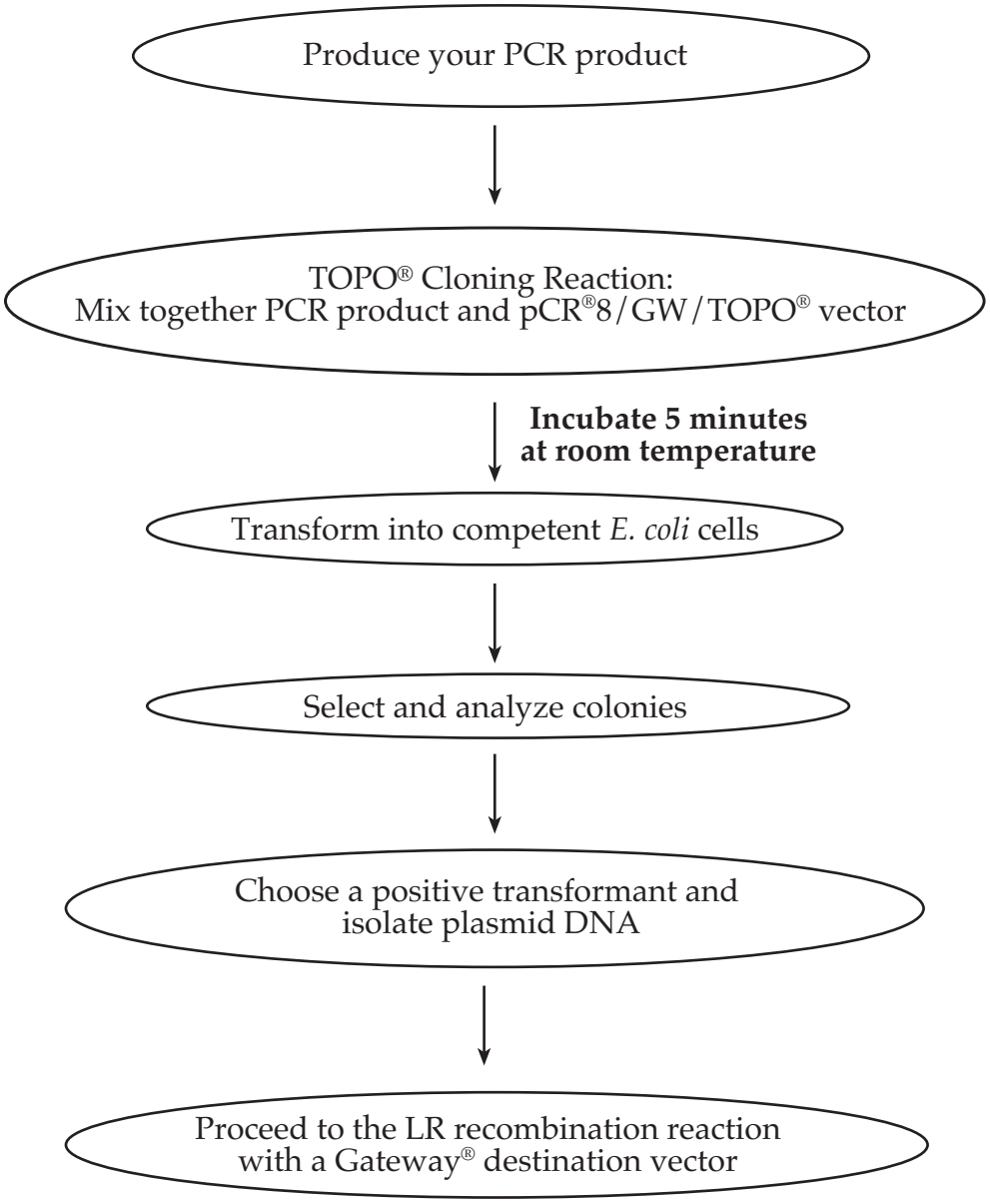
MultiSite Gateway® Technology

The MultiSite Gateway® Technology uses modifications of the site-specific recombination reactions of the Gateway® Technology (see the previous page) to allow simultaneous cloning of multiple DNA fragments in a defined order and orientation. The MultiSite Gateway® Three-Fragment Vector Construction Kit available from Invitrogen (Catalog no. 12537-023) facilitates simultaneous cloning of DNA fragments in three entry vectors to create your own expression clone. For more information about the MultiSite Gateway® Technology and the MultiSite Gateway® Three-Fragment Vector Construction Kit, refer to the MultiSite Gateway® Three-Fragment Vector Construction Kit manual, which is available for downloading from our Web site or by contacting Technical Service.

Experimental Outline

Flow Chart

The flow chart below describes the general steps required to produce and TOPO® Clone your *Taq*-amplified PCR product.


Produce your PCR product
TOPO® Cloning Reaction:
Mix together PCR product and pCR®8/GW/TOPO® vector
Incubate 5 minutes
at room temperature
Transform into competent *E. coli* cells
Select and analyze colonies
Choose a positive transformant and
isolate plasmid DNA
Proceed to the LR recombination reaction
with a Gateway® destination vector

Methods

Designing PCR Primers

Introduction

Before you may use the pCR®8/GW/TOPO® TA Cloning® Kit, you must first design PCR primers and produce your PCR product. Guidelines are provided in this section to help you design PCR primers.

Factors to Consider

It is important to properly design your PCR primers to ensure that you obtain the PCR product you need for your studies. Consider the following when designing your PCR primers:

- If you plan to transfer your PCR product into a Gateway® destination vector for downstream expression studies, remember to include the sequences required for proper translation initiation and termination of your PCR product.
- If you wish to fuse your PCR product to an N- or C-terminal tag after recombination of your entry clone with a Gateway® destination vector, remember to design your PCR primers such that your PCR product will be in frame with the appropriate tag (see **Tips** below). Make sure that the PCR product includes or lacks a Kozak consensus sequence or stop codon, as appropriate to permit proper expression of your recombinant protein. **Note that the first three base pairs of the PCR product will constitute a functional codon.**

Use the diagram on the next page to help you design your PCR primers and your PCR strategy.

Tips

If you wish to fuse your PCR product to an N- or C-terminal tag after recombination of your entry clone with a destination vector, use the tips below as appropriate to design your forward or reverse PCR primer.

Tip 1: To fuse your PCR product in frame with an N-terminal tag after recombination of your entry clone with a destination vector, keep the -AAA-AAA- triplets in the *att*L1 site in frame with the translation reading frame of the fusion protein (see bolded nucleotides in the diagram on the next page).

Tip 2: To fuse your PCR product in frame with a C-terminal tag after recombination of your entry clone with a destination vector, keep the -TTT-GTA (TAC-AAA on the complementary strand) triplets in the *att*L2 site in frame with the translation reading frame of the fusion protein (see bolded nucleotides in the diagram on the next page).

Important

When synthesizing PCR primers, **do not** add 5´ phosphates to the primers as this will prevent the synthesized PCR product from ligating into the pCR®8/GW/TOPO® vector.

continued on next page

Designing PCR Primers, continued

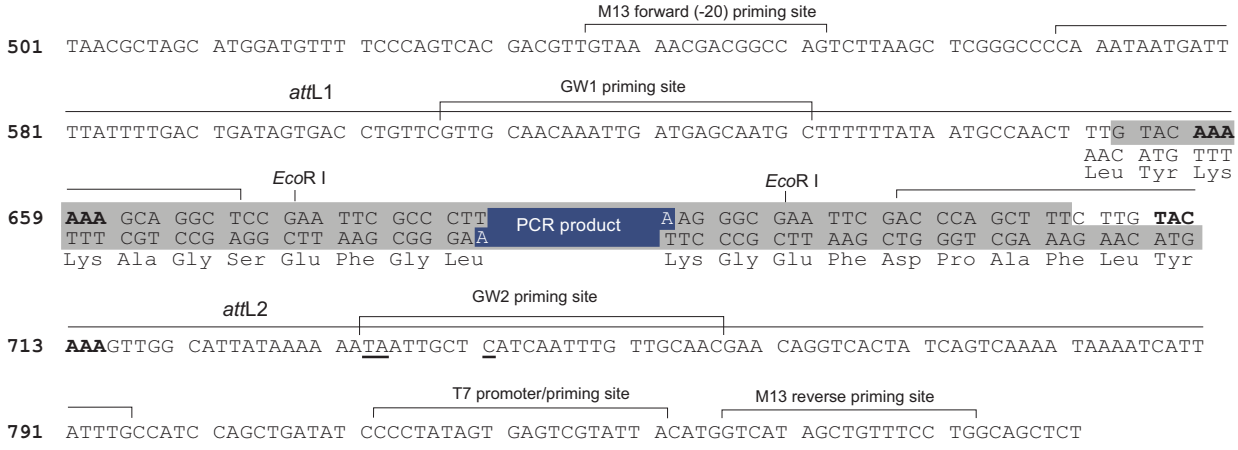
TOPO® Cloning Site for pCR®8/GW/TOPO®

Use the diagram below to help you design PCR primers and produce your PCR product for TOPO® Cloning into pCR®8/GW/TOPO®.

Features of the TOPO® Cloning Region:

- Restriction sites are labeled to indicate the actual cleavage site.
- The primer binding sites for the GW1 and GW2 primers included with the kit are labeled. The nucleotides that were mutated in the *att*L2 site to facilitate sequencing using the GW2 primer are underlined.
- The shaded region corresponds to the DNA sequences that will be transferred from the clone into the Gateway® destination vector following LR recombination.
- If you plan to fuse your PCR product in frame with an N- or C-terminal tag after recombination with a destination vector, remember to keep the translation reading frame of the fusion protein in frame with the triplets indicated in bold, as appropriate.

The sequence of pCR®8/GW/TOPO® is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 27). For more information about pCR®8/GW/TOPO®, see pages 24-25.


M13 forward (-20) priming site
501 TAACGCTAGC ATGGATGTTT TCCCAGTCAC GACGTTGTAA AACGACGGCC AGTCTTAAGC TCGGGCCCCA AATAATGATT
attL1
GW1 priming site
581 TTATTTTGAC TGATAGTGAC CTGTTCGTTG CAACAAATTG ATGAGCAATG CTTTTTTATA ATGCCAACT TTG TAC AAA
AAC ATG TTT
Leu Tyr Lys
EcoR I
EcoR I
659 AAA GCA GGC TCC GAA TTC GCC CTT PCR product AAG GGC GAA TTC GAC CCA GCT TTC TTG TAC
TTT CGT CCG AGG CTT AAG CGG GAA TTC CCG CTT AAG CTG GGT CGA AAG AAC ATG
Lys Ala Gly Ser Glu Phe Gly Leu Lys Gly Glu Phe Asp Pro Ala Phe Leu Tyr
attL2
GW2 priming site
713 AAAGTTGG CATTATAAAA AATAATTGCT CATCAATTTG TTGCAACGAA CAGGTCACTA TCAGTCAAAA TAAAATCATT
T7 promoter/priming site
M13 reverse priming site
791 ATTTGCCATC CAGCTGATAT CCCCTATAGT GAGTCGTATT ACATGGTCAT AGCTGTTTCC TGGCAGCTCT

Note

If you have used other Gateway® entry vectors, note that the sequences of the recombination regions may vary slightly, but the mechanism of recombination remains the same.

Producing PCR Products

Introduction

Once you have synthesized appropriate PCR primers, you may use the primers and a suitable DNA polymerase to produce your PCR product. **Remember that your PCR product must have single 3′ A-overhangs.**

Materials Supplied by the User

You will need the following reagents and equipment for PCR. **Note:** dNTPs (adjusted to pH 8) are provided in the kit.

- *Taq* polymerase or other suitable DNA polymerase

  Note: For improved specificity and higher yields, we recommend using Platinum® *Taq* DNA Polymerase available from Invitrogen (see page x for ordering information) to generate your PCR product.
- Thermocycler
- DNA template and primers to produce the PCR product

Polymerase Mixtures

You may use a polymerase mixture containing *Taq* polymerase and a proofreading polymerase to produce your PCR product; however, the mixture must contain a ratio of *Taq* polymerase:proofreading polymerase in excess of 10:1 to ensure the presence of 3′ A-overhangs on the PCR product. We recommend using Platinum® *Taq* DNA Polymerase High Fidelity available from Invitrogen (see page x for ordering information).

If you use polymerase mixtures that do not have enough *Taq* polymerase or a proofreading polymerase only, you may add 3′ A-overhangs to your PCR product using the method on page 23.

Producing PCR Products

1. Set up the following 50 µl PCR reaction. Use less DNA if you are using plasmid DNA as a template and more DNA if you are using genomic DNA as a template. Use the cycling parameters suitable for your primers and template. Be sure to include a 7 to 30 minute extension at 72°C after the last cycle to ensure that all PCR products are full-length and 3′ adenylated.

DNA Template	10-100 ng
10X PCR Buffer	5 µl
dNTP Mix (50 mM)	0.5 µl
PCR primers (100-200 ng each)	1 µM each
Water	add to a final volume of 49 µl
Taq Polymerase (1 U/µl)	1 µl
Total volume	50 µl

2. Use agarose gel electrophoresis to verify the quality of your PCR product. You should see a single, discrete band of the correct size. If you do not see a single band, refer to the **Note** on the next page.

continued on next page

Producing PCR Products, continued

Note

If you do not obtain a single, discrete band from your PCR, try the following:

- Optimize your PCR to eliminate multiple bands and smearing (Innis *et al.*, 1990). The PCR Optimizer™ Kit available from Invitrogen (Catalog no. K1220-01) incorporates many of the recommendations found in this reference. For more information, refer to our Web site (www.invitrogen.com) or contact Technical Service (see page 27).
- Gel-purify your fragment using one of the methods on pages 21-22. Take special care to avoid sources of nuclease contamination.

Setting Up the TOPO® Cloning Reaction

Introduction

Once you have produced the desired PCR product, you are ready to TOPO® Clone it into the pCR®8/GW/TOPO® vector and transform the recombinant vector into One Shot® competent *E. coli*. You should have everything you need set up and ready to use to ensure that you obtain the best possible results. We suggest that you read this section and the section entitled **Transforming One Shot® Competent *E. coli*** (pages 11-13) before beginning. If this is the first time you have TOPO® Cloned, perform the control reactions on pages 19-20 in parallel with your samples.

Note

We have found that including salt (200 mM NaCl, 10 mM $MgCl_2$) in the TOPO® Cloning reaction can increase the number of transformants 2- to 3-fold. In addition, incubating the reaction mixture for greater than 5 minutes in the presence of salt can also increase the number of transformants. This is in contrast to earlier experiments **without salt** where the number of transformants decreases as the incubation time increases beyond 5 minutes.

Including salt in the TOPO® Cloning reaction allows for longer incubation times because it prevents topoisomerase I from rebinding and potentially nicking the DNA after ligating the PCR product and dissociating from the DNA. The result is more intact molecules, leading to higher transformation efficiencies.

Using Salt Solution in the TOPO® Cloning Reaction

You will perform TOPO® Cloning in a reaction buffer containing salt (*i.e.* using the stock salt solution provided in the kit). **Note that the amount of salt added to the TOPO® Cloning reaction varies depending on whether you plan to transform chemically competent cells (provided) or electrocompetent cells (see page x for ordering information).**

- If you are transforming chemically competent *E. coli*, use the stock Salt Solution as supplied and set up the TOPO® Cloning reaction as directed on the next page.
- If you are transforming electrocompetent *E. coli*, the amount of salt in the TOPO® Cloning reaction **must be reduced** to 50 mM NaCl, 2.5 mM $MgCl_2$ to prevent arcing during electroporation. Dilute the stock Salt Solution 4-fold with water to prepare a 300 mM NaCl, 15 mM $MgCl_2$ Dilute Salt Solution. Use the Dilute Salt Solution to set up the TOPO® Cloning reaction as directed on the next page.

continued on next page

Setting Up the TOPO® Cloning Reaction, continued

Materials Needed

You should have the following materials on hand before beginning:

- Your PCR product (freshly prepared)
- pCR®8/GW/TOPO® vector (supplied with the kit, Box 1; keep at -20°C until use)
- Salt Solution (supplied with the kit, Box 1) or Dilute Salt Solution as appropriate
- Water (supplied with the kit, Box 1)

Performing the TOPO® Cloning Reaction

Use the procedure below to perform the TOPO® Cloning reaction. Set up the TOPO® Cloning reaction using the reagents in the order shown, and depending on whether you plan to transform chemically competent *E. coli* or electrocompetent *E. coli.*

Note: The red color of the TOPO® vector solution is normal and is used to visualize the solution.

Reagent*	Chemically Competent *E. coli*	Electrocompetent *E. coli*
Fresh PCR product	0.5 to 4 µl	0.5 to 4 µl
Salt Solution	1 µl	--
Dilute Salt Solution (1:4)	--	1 µl
Water	add to a final volume of 5 µl	add to a final volume of 5 µl
TOPO® vector	1 µl	1 µl
Final volume	6 µl	6 µl

*Store all reagents at –20°C when finished. Salt solution and water can be stored at room temperature or +4°C.

1. Mix reaction gently and incubate for 5 minutes at room temperature (22-23°C).

   Note: For most applications, 5 minutes will yield a sufficient number of colonies for analysis. Depending on your needs, the length of the TOPO® Cloning reaction can be varied from 30 seconds to 30 minutes. For routine subcloning of PCR products, 30 seconds may be sufficient. For large PCR products (> 1 kb) or if you are TOPO® Cloning a pool of PCR products, increasing the reaction time may yield more colonies.

2. Place the reaction on ice and proceed to **Transforming One Shot® Competent *E. coli***, next page.

   Note: You may store the TOPO® Cloning reaction at –20°C overnight.

Transforming One Shot® Competent *E. coli*

Introduction

Once you have performed the TOPO® Cloning reaction, you will transform your pCR®8/GW/TOPO® construct into competent *E. coli*. One Shot® TOP10 or Mach1™-T1^R Chemically Competent *E. coli* (Box 2) are included with the kit to facilitate transformation. You may also transform electrocompetent cells, if desired (see page x for ordering information). Protocols to transform chemically competent or electrocompetent *E. coli* are provided in this section.

Selecting a One Shot® Chemical Transformation Protocol

Two protocols are provided to transform One Shot® TOP10 or Mach1™-T1^R chemically competent *E. coli*. Consider the following factors and choose the protocol that best suits your needs.

If you wish to…	Then use the…
maximize the number of transformants	regular chemical transformation protocol, page 12
clone large PCR products (>1000 bp)	
obtain transformants as quickly as possible	rapid chemical transformation protocol, page 13 **Note:** This procedure is less efficient; the total number of transformants obtained may be lower than that obtained with the regular chemical transformation protocol

Materials Needed

In addition to general microbiological supplies (*i.e.* plates, spreaders), you will need the following reagents and equipment:

- TOPO® Cloning reaction (from Step 2, previous page)
- One Shot® TOP10 or Mach1™-T1^R chemically competent *E. coli* (supplied with the kit, Box 2)
- S.O.C. Medium (included with the kit, Box 2)
- pUC19 positive control (to verify transformation efficiency, if desired, Box 2)
- 42°C water bath (or electroporator with cuvettes, optional)
- 15 ml sterile, snap-cap plastic culture tubes (for electroporation only)
- LB plates containing 100 µg/ml spectinomycin (two for each transformation; see page 26 for a recipe to prepare spectinomycin)
- LB plates containing 100 µg/ml ampicillin (if transforming pUC19 control)
- 37°C shaking and non-shaking incubator

Note

There is no blue-white screening for the presence of inserts. Most transformants will contain recombinant plasmids with the PCR product of interest cloned into the vector. The GW1 and GW2 primers are included in the kit to allow you to sequence across an insert in the TOPO® Cloning site to confirm orientation and reading frame.

continued on next page

Transforming One Shot® Competent *E. coli*, continued

Preparing for Transformation

For each transformation, you will need one vial of One Shot® competent cells and two selective plates.

- Equilibrate a water bath to 42°C (for chemical transformation) or set up your electroporator if you are using electrocompetent *E. coli*.
- Warm the vial of S.O.C. Medium from Box 2 to room temperature.
- Warm LB plates containing 100 µg/ml spectinomycin at 37°C for 30 minutes (see **Important Note** below). If you are including the pUC19 positive control, prewarm LB plates containing 100 µg/ml ampicillin as well.
- Thaw **on ice** one vial of One Shot® cells for each transformation.

Important

If you are performing the rapid chemical transformation protocol, it is **essential** that you prewarm your LB plates containing 100 µg/ml spectinomycin prior to spreading.

One Shot® Chemical Transformation Protocol

Use the following protocol to transform One Shot® TOP10 or Mach1™-T1^R chemically competent *E. coli*.

1. Add 2 µl of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, page 10 into a vial of One Shot® Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down**.

   Note: If you are transforming the pUC19 control plasmid, use 10 pg (1 µl).
2. Incubate on ice for 5 to 30 minutes.

   Note: Longer incubations on ice seem to have a minimal effect on transformation efficiency. The length of the incubation is at the user's discretion.
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice.
5. Add 250 µl of room temperature S.O.C. Medium.
6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
7. Spread 10-50 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. To ensure even spreading of small volumes, add 20 µl of S.O.C. Medium. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
8. An efficient TOPO® Cloning reaction should produce several hundred colonies. Pick 10 colonies for analysis (see **Analyzing Transformants**, page 14).

continued on next page

Transforming One Shot® Competent *E. coli*, continued

Rapid One Shot® Chemical Transformation Protocol

Use the alternative protocol below to rapidly transform One Shot® TOP10 or Mach1™-T1^R chemically competent *E. coli*. Before beginning, make sure to pre-warm LB agar plates containing 100 µg/ml spectinomycin at 37°C for 30 minutes.

1. Add **4 µl** of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, page 10 into a vial of One Shot® Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down**.
2. Incubate on ice for 5 minutes.
3. Spread **50 µl** of cells on a prewarmed selective plate and incubate overnight at 37°C.
4. An efficient TOPO® Cloning reaction should produce several hundred colonies. Pick 10 colonies for analysis (see **Analyzing Transformants**, page 14).

One Shot® Electroporation Protocol

Use ONLY electrocompetent cells for electroporation to avoid arcing. Do not use the One Shot® TOP10 or Mach1™-T1^R chemically competent cells for electroporation.

1. Add 2 µl of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, page 10 into a sterile microcentrifuge tube containing 50 µl of electrocompetent *E. coli* and mix gently. **Do not mix by pipetting up and down. Avoid formation of bubbles.** Transfer the cells to a 0.1 cm cuvette.
2. Electroporate your samples using your own protocol and your electroporator.

   Note: If you have problems with arcing, see below.
3. Immediately add 250 µl of room temperature S.O.C. Medium.
4. Transfer the solution to a 15 ml snap-cap tube (*i.e.* Falcon) and shake for at least 1 hour at 37°C to allow expression of the spectinomycin resistance gene.
5. Spread 10-50 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. To ensure even spreading of small volumes, add 20 µl of S.O.C. Medium. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
6. An efficient TOPO® Cloning reaction should produce several hundred colonies. Pick 10 colonies for analysis (see **Analyzing Transformants**, page 14).

To prevent arcing of your samples during electroporation, the volume of cells should be between 50 and 80 µl (0.1 cm cuvettes) or 100 to 200 µl (0.2 cm cuvettes).

If you experience arcing during transformation, try one of the following suggestions:

- Reduce the voltage normally used to charge your electroporator by 10%.
- Reduce the pulse length by reducing the load resistance to 100 ohms.
- Ethanol precipitate the TOPO® Cloning reaction and resuspend in water prior to electroporation.

Analyzing Transformants

Analyzing Positive Clones

1. Pick 2-6 colonies and culture them overnight in LB or SOB medium containing 100 µg/ml spectinomycin.

   Note: If you transformed One Shot® Mach1™-T1^R competent *E. coli*, you may inoculate overnight-grown colonies and culture them for only 4 hours in pre-warmed LB medium containing 100 µg/ml spectinomycin before isolating plasmid. For optimal results, we recommend inoculating as much of a single colony as possible.

2. Isolate plasmid DNA using PureLink™ Quick Plasmid Miniprep Kit (supplied with cat. no. K2520-02 or available separately, page x). The plasmid isolation protocol is included in the manual supplied with the PureLink™ Quick Plasmid Miniprep Kit and is also available for downloading from www.invitrogen.com. Other kits for plasmid DNA purification are also suitable for use.

3. Analyze the plasmids by restriction analysis or PCR to confirm the presence and correct orientation of the insert.

   Note: pCR®8/GW/TOPO® contains *Eco*R I sites flanking the TOPO® Cloning site. You may use *Eco*R I digestion to check for the presence of inserts, if desired.

Sequencing

Once you have identified the correct clone(s), you may sequence your construct to confirm that your gene is cloned in the correct orientation. The GW1 and GW2 primers are included in the kit to help you sequence your insert (see the diagrams on page 6 for the location of the priming sites in pCR®8/GW/TOPO® vector). For the complete sequence of the pCR®8/GW/TOPO® vector, see our Web site (www.invitrogen.com) or call Technical Service (see page 27).

Important

The GW1 and GW2 primer sites are located less than 55 nucleotides from the PCR product insertion site, and fall within the *att*L1 and *att*L2 sites, respectively of pCR®8/GW/TOPO®. Although Invitrogen offers other Gateway® entry vectors containing *att*L1 and *att*L2 sites, the GW1 and GW2 primers are only suitable for use in sequencing inserts cloned into pCR®8/GW/TOPO®. This is because three nucleotides within the *att*L2 site in pCR®8/GW/TOPO® have been mutated (see the diagram on page 6 for details). **These mutations allow GW1 and GW2 primer-based sequencing, but do not affect the LR recombination efficiency.**

Long-Term Storage

Once you have identified the correct clone, be sure to purify the colony and make a glycerol stock for long-term storage. We recommend that you store a stock of plasmid DNA at -20°C.

1. Streak the original colony out for single colonies on an LB plate containing 100 µg/ml spectinomycin.
2. Isolate a single colony and inoculate into 1-2 ml of LB containing 100 µg/ml spectinomycin.
3. Grow until culture reaches stationary phase.
4. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
5. Store at -80°C.

Guidelines to Perform the LR Recombination Reaction

Introduction

Once you have obtained your entry clone, you may:

- Perform an LR recombination reaction using Gateway® LR Clonase™ II enzyme mix (see page x for ordering information) to transfer your gene of interest from the pCR®8/GW/TOPO® construct into any Gateway® destination vector of choice to generate an expression clone.
- Perform a MultiSite Gateway® LR recombination reaction with 5′ and 3′ entry clones, the appropriate MultiSite Gateway® destination vector, and LR Clonase™ Plus enzyme mix (see page x for ordering information) to generate an expression clone.

General guidelines are provided below.

Important

For most applications, we recommend performing the LR recombination reaction or the MultiSite Gateway® LR recombination reaction using:

- Supercoiled entry clone(s)
- Supercoiled destination vector

Destination Vectors

A large selection of Gateway® destination vectors is available from Invitrogen to facilitate expression of your gene of interest in virtually any protein expression system. For more information about the vectors available, see our Web site (www.invitrogen.com) or call Technical Service (see page 27). Manuals supporting all of the destination vectors are available for downloading from our Web site or by contacting Technical Service.

E. coli Host

Once you have performed the LR recombination reaction or the MultiSite Gateway® LR recombination reaction, you will transform the reaction mixture into competent *E. coli* and select for expression clones. You may use any *rec*A, *end*A *E. coli* strain including TOP10, Mach1™-T1^R, DH5α™, DH10B™, or equivalent for transformation. Do not transform the Gateway® or MultiSite Gateway® LR reaction mixture into *E. coli* strains that contain the F′ episome (*e.g.* TOP10F′). These strains contain the *ccd*A gene and will prevent negative selection with the *ccd*B gene.

continued on next page

Guidelines to Perform the LR Recombination Reaction

Performing the LR Recombination Reaction

To perform the Gateway® LR recombination reaction, you will need:

- Purified plasmid DNA of the entry clone containing your gene of interest
- A destination vector of choice
- LR Clonase™ II enzyme mix (see **Recommendation** below and page x for ordering information)
- 2 µg/µl Proteinase K solution (supplied with the LR Clonase™ II enzyme mix)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- Appropriate chemically competent *E. coli* host and growth media for expression
- Appropriate selective plates

For instructions to perform the LR recombination reaction, refer to the LR Clonase™ II Enzyme Mix manual or to the manual for the destination vector you are using.

To catalyze the LR recombination reaction, we recommend using Gateway® LR Clonase™ II Enzyme Mix. The LR Clonase™ II enzyme mix combines the proprietary enzyme formulation and 5X LR Reaction Buffer previously supplied by Invitrogen as separate components in LR Clonase™ enzyme mix (Catalog no. 11791-019) into an optimized single-tube format for easier set-up of the LR recombination reaction.

Note: You may perform the LR recombination reaction using LR Clonase™ enzyme mix, if desired. Follow the instructions included with the product to perform the LR recombination reaction.

Performing the MultiSite Gateway® LR Recombination Reaction

Before you can perform the MultiSite Gateway® LR recombination reaction, you will first need to generate 5′ and 3′ entry clones using Invitrogen's MultiSite Gateway® Three-Fragment Vector Construction Kit (Catalog no. 12537-023). Once you have generated the 5′ and 3′ entry clones, you will use the 5′ and 3′ entry clones, the entry clone containing your gene of interest, and the other reagents supplied in the MultiSite Gateway® Three-Fragment Vector Construction Kit (including LR Clonase™ Plus enzyme mix and the pDEST™R4-R3 destination vector) in a MultiSite Gateway® LR recombination reaction to generate an expression clone.

For instructions to generate 5′ and 3′ entry clones and to perform the MultiSite Gateway® LR recombination reaction, refer to the MultiSite Gateway® Three-Fragment Vector Construction Kit manual.

Troubleshooting

TOPO® Cloning Reaction and Transformation

The table below lists some potential problems and possible solutions that may help you troubleshoot the TOPO® Cloning and transformation reactions. To help evaluate your results, we recommend that you perform the control reactions (see pages 19-20) in parallel with your samples.

Problem	Reason	Solution
Few or no colonies obtained from sample reaction **and** the transformation control gave colonies	Incomplete extension during PCR	Include a final extension step of 7 to 30 minutes during PCR. Longer PCR products will need a longer extension time.
	Excess (or overly dilute) PCR product used in the TOPO® Cloning reaction	Reduce (or concentrate) the amount of PCR product.
	PCR primers contain 5′ phosphates	Do not add 5′ phosphates to your PCR primers.
	Used a proofreading polymerase or a *Taq*/proofreading polymerase mixture for PCR	• Use *Taq* polymerase or another DNA polymerase that leaves 3′ A-overhangs to produce your PCR product. • Add 3′ A-overhangs to your blunt PCR product by incubating with *Taq* polymerase (see page 23).
	Large PCR product	• Increase the amount of PCR product used in the TOPO® Cloning reaction. • Increase the incubation time of the TOPO® Cloning reaction from 5 minutes to 30 minutes. • Gel-purify the PCR product to remove primer-dimers and other artifacts.
	PCR reaction contains artifacts (*i.e.* does not run as a single band on an agarose gel)	• Optimize your PCR conditions. • Gel-purify your PCR product.
	Cloning large pool of PCR products or a toxic gene	Increase the incubation time of the TOPO® reaction from 5 minutes to 30 minutes.

continued on next page

Troubleshooting, continued

TOPO® Cloning Reaction and Transformation, continued

Problem	Reason	Solution
Few or no colonies obtained from sample reaction **and** the transformation control gave colonies, continued	PCR product does not contain sufficient 3′ A-overhangs even though you used *Taq* polymerase	• Increase the final extension time to ensure that all 3′ ends are adenylated. • *Taq* polymerase is most efficient at adding a non-template 3′ A next to a C, and less efficient at adding a nontemplate 3′ A next to another A. You may have to re-design your primers so that they contain a 5′ G instead of a 5′ T (Brownstein *et al.*, 1996).
Large number of incorrect inserts cloned	PCR cloning artifacts	• Gel-purify your PCR product to remove primer-dimers and smaller PCR products. • Optimize your PCR conditions. • Include a final extension step of 7 to 30 minutes during PCR. Longer PCR products will need a longer extension time.
Few or no colonies obtained from sample reaction **and** the transformation control gave **no** colonies	One Shot® competent *E. coli* stored incorrectly	Store One Shot® competent *E. coli* at -80°C. If you are using another *E. coli* strain, follow the manufacturer's instructions.
	Did not perform the 1 hour grow-out period before plating the transformation mixture	After the heat-shock step, add S.O.C. Medium and incubate the transformation mixture for 1 hour at 37°C before plating.
	Insufficient amount of *E. coli* plated	Increase the amount of *E. coli* plated.
	Transformants plated on selective plates containing the wrong antibiotic	Use the appropriate antibiotic for selection.

Appendix

Performing the Control Reactions

Introduction

We recommend performing the following control TOPO® Cloning reactions the first time you use the kit to help you evaluate your results. Performing the control reactions involves producing a control PCR product containing the *lac* promoter and the LacZα fragment using the reagents included in the kit. Successful TOPO® Cloning of the control PCR product in either direction will yield blue colonies on LB agar plates containing spectinomycin and X-gal.

Before Starting

For each transformation, prepare two LB plates containing 100 µg/ml spectinomycin and X-gal (see page 26 for recipes).

Producing the Control PCR Product

Use the procedure below to produce the 500 bp control PCR product using *Taq* polymerase.

1. In a 0.5 ml microcentrifuge tube, set up the following 50 µl PCR:

Reagent	Amount
Control DNA Template (50 ng)	1 µl
10X PCR Buffer	5 µl
dNTP Mix	0.5 µl
Control PCR Primers (0.1 µg/µl each)	1 µl
Water	41.5 µl
Taq polymerase (1 U/µl)	1 µl
Total volume	50 µl

2. Overlay with 70 µl (1 drop) of mineral oil, if required.
3. Amplify using the following cycling parameters:

Step	Time	Temperature	Cycles
Initial Denaturation	2 minutes	94°C	1X
Denaturation	1 minute	94°C	25X
Annealing	1 minute	60°C	
Extension	1 minute	72°C	
Final Extension	7 minutes	72°C	1X

4. Remove 10 µl from the reaction and analyze by agarose gel electrophoresis. A discrete 500 bp band should be visible. Proceed to the **Control TOPO® Cloning Reactions**, next page.

continued on next page

Performing the Control Reactions, continued

Control TOPO® Cloning Reactions

Using the control PCR product produced on the previous page and the pCR®8/GW/TOPO® vector, set up two 6 µl TOPO® Cloning reactions as described below.

1. Set up control TOPO® Cloning reactions:

Reagent	"Vector Only"	"Vector + PCR Insert"
Water	4 µl	3 µl
Salt Solution	1 µl	1 µl
Control PCR Product	--	1 µl
pCR®8/GW/TOPO® vector	1 µl	1 µl
Total volume	6 µl	6 µl

2. Incubate at room temperature for **5 minutes** and place on ice.
3. Transform 2 µl of each reaction into separate vials of One Shot® competent cells using the procedure on page 12.
4. Spread 10-50 µl of each transformation mix onto LB plates containing 100 µg/ml spectinomycin and X-gal. When plating small volumes, add 20 µl of S.O.C. Medium to ensure even spreading. Be sure to plate two different volumes to ensure that at least one plate has well-spaced colonies.
5. Incubate overnight at 37°C.

What You Should See

The "vector + PCR insert" reaction should be produce hundreds of colonies. Greater than 95% of these will be blue.

The "vector only" reaction should yield very few colonies (< 5% of the vector + PCR insert plate) and these should be white.

Transformation Control

pUC19 plasmid is included to check the transformation efficiency of the One Shot® TOP10 or Mach1™-T1^R competent cells. Transform one vial of One Shot® TOP10 or Mach1™-T1^R cells with 10 pg of pUC19 using the protocol on page 12. Plate 10 µl of the transformation mixture plus 20 µl of S.O.C. Medium on LB plates containing 100 µg/ml ampicillin. Transformation efficiency should be ≥ 1 x 10^9 cfu/µg DNA.

Gel Purifying PCR Products

Introduction

Smearing, multiple banding, primer-dimer artifacts, or large PCR products (>3 kb) may necessitate gel purification. If you wish to purify your PCR product, be extremely careful to remove all sources of nuclease contamination. There are many protocols to isolate DNA fragments or remove oligonucleotides. Refer to *Current Protocols in Molecular Biology*, Unit 2.6 (Ausubel *et al.*, 1994) for the most common protocols. Two simple protocols are provided below.

Using the PureLink™ Quick Gel Extraction Kit

The PureLink™ Quick Gel Extraction Kit (page x) allows you to rapidly purify PCR products from regular agarose gels.

1. Equilibrate a water bath or heat block to 50°C.
2. Cut the area of the gel containing the desired DNA fragment using a clean, sharp blade. Minimize the amount of surrounding agarose excised with the fragment. Weigh the gel slice.
3. Add Gel Solubilization Buffer (GS1) supplied in the kit as follows:
   - For ≤2% agarose gels, place up to 400 mg gel into a sterile, 1.5-ml polypropylene tube. Divide gel slices exceeding 400 mg among additional tubes. Add 30 µl Gel Solubilization Buffer (GS1) for every 10 mg of gel.
   - For >2% agarose gels, use sterile 5-ml polypropylene tubes and add 60 µl Gel Solubilization Buffer (GS1) for every 10 mg of gel.
4. Incubate the tube at 50°C for 15 minutes. Mix every 3 minutes to ensure gel dissolution. After gel slice appears dissolved, incubate for an **additional** 5 minutes.
5. Preheat an aliquot of TE Buffer (TE) to 65-70°C
6. Place a Quick Gel Extraction Column into a Wash Tube. Pipette the mixture from Step 4, above onto the column. Use 1 column per 400 mg agarose.
7. Centrifuge at >12,000 x g for 1 minute. Discard the flow-through. Place the column back into the Wash Tube.
8. **Optional:** Add 500 µl Gel Solubilization Buffer (GS1) to the column. Incubate at room temperature for 1 minute. Centrifuge at >12,000 x g for 1 minute. Discard the flow-through. Place the column back into the Wash Tube.
9. Add 700 µl Wash Buffer (W9) with ethanol (add 96–100% ethanol to the Wash Buffer according to instructions on the label of the bottle) to the column and incubate at room temperature for 5 minutes. Centrifuge at >12,000 x g for 1 minute. Discard flow-through.
10. Centrifuge the column at >12,000 x g for 1 minute to remove any residual buffer. Place the column into a 1.5 ml Recovery Tube.
11. Add 50 µl **warm** (65-70°C) TE Buffer (TE) to the center of the cartridge. Incubate at room temperature for 1 minute.
12. Centrifuge at >12,000 x g for 2 minutes. *The Recovery Tube contains the purified DNA.* Store DNA at –20°C. Discard the column.
13. Use 4 µl of the purified DNA for the TOPO® Cloning reaction.

continued on next page

Gel Purifying PCR Products, continued

Low-Melt Agarose Method

If you prefer to use low-melt agarose, use the procedure below. Note that gel purification will result in a dilution of your PCR product and a potential loss of cloning efficiency.

1. Electrophorese as much as possible of your PCR reaction on a low-melt agarose gel (0.8 to 1.2%) in TAE buffer.
2. Visualize the band of interest and excise the band.
3. Place the gel slice in a microcentrifuge tube and incubate the tube at 65°C until the gel slice melts.
4. Place the tube at 37°C to keep the agarose melted.
5. Add 4 µl of the melted agarose containing your PCR product to the TOPO® Cloning reaction as described on page 10.
6. Incubate the TOPO® Cloning reaction **at 37°C for 5 to 10 minutes**. This is to keep the agarose melted.
7. Transform 2 to 4 µl directly into One Shot® competent cells using the method on page 12.

Note

The cloning efficiency may decrease with purification of the PCR product (*e.g.* PCR product too dilute). You may wish to optimize your PCR to produce a single band (see **Producing PCR Products**, page 7).

Addition of 3´ A-Overhangs Post-Amplification

Introduction

Direct cloning of DNA amplified by proofreading polymerases into TOPO TA Cloning® vectors is often difficult because proofreading polymerases remove the 3´ A-overhangs necessary for TA Cloning®. Invitrogen has developed a simple method to clone these blunt-ended fragments.

Before Starting

You will need the following items:

- *Taq* polymerase
- A heat block equilibrated to 72°C
- Phenol-chloroform (optional)
- 3 M sodium acetate (optional)
- 100% ethanol (optional)
- 80% ethanol (optional)
- TE buffer (optional)

Procedure

This is just one method for adding 3´ adenines. Other protocols may be suitable.

1. After amplification with a proofreading polymerase, place vials on ice and add 0.7-1 unit of *Taq* polymerase per tube. Mix well. It is not necessary to change the buffer. A sufficient number of PCR products will retain the 3´ A-overhangs.
2. Incubate at 72°C for 8-10 minutes (do not cycle).
3. Place on ice and use immediately in the TOPO® Cloning reaction.

Note: If you plan to store your sample overnight before proceeding with TOPO® Cloning, extract your sample with an equal volume of phenol-chloroform to remove the polymerases. Ethanol-precipitate the DNA and resuspend in TE buffer using the starting volume of the PCR.

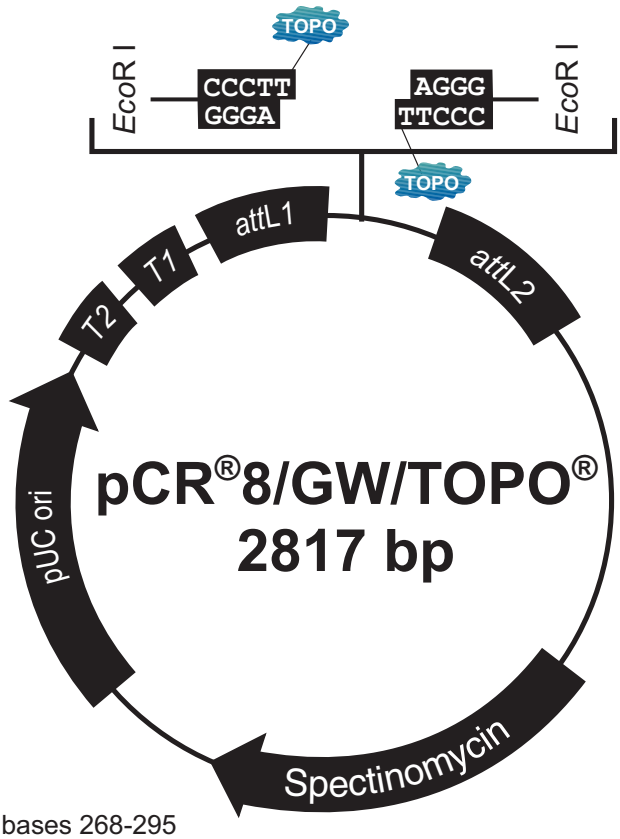
Note

You may also gel-purify your PCR product after amplification with a proofreading polymerase. After purification, add *Taq* polymerase buffer, dATP, and 0.5 unit of *Taq* polymerase. Incubate the reaction for 10-15 minutes at 72°C and use in the TOPO® Cloning reaction.

Map and Features of pCR®8/GW/TOPO®

pCR®8/GW/TOPO® Map

The figure below shows the features of the pCR®8/GW/TOPO® vector. **The complete sequence of pCR®8/GW/TOPO® is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 27).**



Comments for pCR®8/GW/TOPO®
2817 nucleotides

*rrn*B T2 transcription termination sequence: bases 268-295
*rrn*B T1 transcription termination sequence: bases 427-470
M13 forward (-20) priming site: bases 537-552
*att*L1: bases 569-668
GW1 priming site: bases 607-631
TOPO® recognition site 1: bases 678-682
TOPO® recognition site 2: bases 683-687
*att*L2: bases 696-795
GW2 priming site: bases 733-757
T7 Promoter/priming site: 812-831 (c)
M13 reverse priming site: bases 836-852
Spectinomycin promoter: bases 930-1063
Spectinomycin resistance gene (Spn^R): 1064-2074
pUC origin: bases 2141-2814

(c) = complementary sequence

continued on next page

Map and Features of pCR®8/GW/TOPO®, continued

Features of pCR®8/GW/TOPO®

pCR®8/GW/TOPO® (2817 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
*rrn*B T1 and T2 transcription termination sequences	Reduces potential toxicity in *E. coli* by preventing basal expression of the PCR product.
T7 promoter/priming site	Allows *in vitro* transcription, and sequencing through the insert.
M13 forward (-20) priming site	Allows sequencing of the insert.
GW1 priming site	Allows sequencing of the insert.
*att*L1 and *att*L2 sites	Bacteriophage λ-derived recombination sequences that allow recombinational cloning of a gene of interest in the entry construct with a Gateway® destination vector (Landy, 1989).
TOPO® Cloning site	Allows rapid cloning of your *Taq*-amplified PCR product.
GW2 priming site	Allows sequencing of the insert.
M13 reverse priming site	Allows sequencing of the insert.
Spectinomycin promoter	Allows expression of the spectinomycin resistance gene in *E. coli*.
Spectinomycin resistance gene (*aad*A1)	Allows selection of the plasmid in *E. coli* (Liebert *et al.*, 1999).
pUC origin of replication (*ori*)	Allows high-copy replication and maintenance in *E. coli*.

Recipes

LB (Luria-Bertani) Medium and Plates

Composition:

1.0% Tryptone
0.5% Yeast Extract
1.0% NaCl
pH 7.0

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 ml deionized water.
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave on liquid cycle for 20 minutes. Allow solution to cool to ~55°C and add antibiotic, if needed.
4. Store at room temperature or at +4°C.

LB agar plates

1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle for 20 minutes.
3. After autoclaving, cool to ~55°C, add antibiotic and pour into 10 cm plates.
4. Let harden, then invert and store at +4°C, in the dark.
5. To add X-gal to the plate, warm the plate to 37°C. Pipette 40 µl of the 40 mg/ml X-gal stock solution (see below), spread evenly, and let dry for 15 minutes. Protect plates from light.

Spectinomycin

Use this procedure to prepare a 10 mg/ml stock solution of spectinomycin.

Materials Needed

- Spectinomycin dihydrochloride (Sigma, Catalog no. S4014)
- Sterile, deionized water

Procedure

1. Weigh out 50 mg of spectinomycin and transfer to a sterile centrifuge tube.
2. Resuspend the spectinomycin in 5 ml of sterile, deionized water to produce a 10 mg/ml stock solution.
3. Filter-sterilize.
4. Store the stock solution at +4°C for up to 2 weeks. For long-term storage, store at -20°C.

X-Gal Stock Solution

1. Dissolve 400 mg of X-gal in 10 ml dimethylformamide to prepare a 40 mg/ml stock solution.
2. Store at -20°C, protected from light.

Technical Service

Web Resources

Visit the Invitrogen Web site at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical service contact information
- Access to the Invitrogen Online Catalog
- Additional product information and special offers

Contact Us

For more information or technical assistance, call, write, fax, or email. Additional international offices are listed on our Web page (www.invitrogen.com).

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MSDSs

MSDSs (Material Safety Data Sheets) are available on our website at www.invitrogen.com/msds.

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Purchaser Notification

Introduction

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Information for European Customers

The Mach1™-T1^R *E. coli* strain is genetically modified to carry the *lac*ZΔM15 *hsd*R *lac*X74 *rec*A *end*A *ton*A genotype. As a condition of sale, use of this product must be in accordance with all applicable local legislation and guidelines including EC Directive 90/219/EEC on the contained use of genetically modified organisms.

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continued on next page

Purchaser Notification, continued

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Gateway® Clone Distribution Policy

For additional information about Invitrogen's policy for the use and distribution of Gateway® clones, see **Gateway® Clone Distribution Policy**, next page.

Gateway® Clone Distribution Policy

Introduction

The information supplied in this section is intended to provide clarity concerning Invitrogen's policy for the use and distribution of cloned nucleic acid fragments, including open reading frames, created using Invitrogen's commercially available Gateway® Technology.

Gateway® Entry Clones

Invitrogen understands that Gateway® entry clones, containing *att*L1 and *att*L2 sites, may be generated by academic and government researchers for the purpose of scientific research. Invitrogen agrees that such clones may be distributed for scientific research by non-profit organizations and by for-profit organizations without royalty payment to Invitrogen.

Gateway® Expression Clones

Invitrogen also understands that Gateway® expression clones, containing *att*B1 and *att*B2 sites, may be generated by academic and government researchers for the purpose of scientific research. Invitrogen agrees that such clones may be distributed for scientific research by academic and government organizations without royalty payment to Invitrogen. Organizations other than academia and government may also distribute such Gateway® expression clones for a nominal fee ($10 per clone) payable to Invitrogen.

Additional Terms and Conditions

We would ask that such distributors of Gateway® entry and expression clones indicate that such clones may be used only for research purposes, that such clones incorporate the Gateway® Technology, and that the purchase of Gateway® Clonase™ from Invitrogen is required for carrying out the Gateway® recombinational cloning reaction. This should allow researchers to readily identify Gateway® containing clones and facilitate their use of this powerful technology in their research. Use of Invitrogen's Gateway® Technology, including Gateway® clones, for purposes other than scientific research may require a license and questions concerning such commercial use should be directed to Invitrogen's licensing department at 760-603-7200.

Product Qualification

Introduction

This section describes the criteria used to qualify the components of the pCR®8/GW/TOPO® TA Cloning® Kit.

PCR®8/GW Vector

Prior to adaptation with topoisomerase I, the supercoiled pCR®8/GW vector is qualified by:

- Performing restriction enzyme digestion to verify its structure.
- Performing an LR recombination reaction with a Gateway® destination vector to confirm its functionality.

TOPO® Cloning Efficiency

After adaptation with topoisomerase I, each lot of pCR®8/GW/TOPO® vector is functionally qualified using the control reagents included in the kit. Under conditions described on pages 19-20, a 500 bp control PCR product is amplified, TOPO® Cloned into the pCR®8/GW/TOPO® vector, and transformed into One Shot® TOP10 or Mach1™-T1^R chemically competent *E. coli* included with the kit.

Each lot of vector should yield greater than 95% cloning efficiency.

Primers

Primers are lot-qualified by DNA sequencing experiments using the dideoxy chain termination technique.

One Shot® Chemically Competent *E. coli*

One Shot® TOP10 and Mach1™-T1^R chemically competent cells are tested for transformation efficiency using the control plasmid included in the kit. Transformed cultures are plated on LB plates containing 100 µg/ml ampicillin and the transformation efficiency is calculated. Test transformations are performed in duplicate. Transformation efficiency should be greater than 1×10^9 cfu/µg plasmid DNA.

In addition, untransformed cells are tested for the appropriate antibiotic sensitivity and lack of phage contamination.

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